CHARACTERIZATION OF THE ANTITUMOR EFFECTS OF INTERFERONS ON PROSTATE CANCER CELLS

By

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Interferons (IFNs) function as important cytokines with a broad range of effects on cells of various origins. Included in these effects are potent antitumor capabilities. I investigated the antitumor effects of both type I and type II IFNs on a human prostate cancer cell line DU145. DU145 cells are a prostatic adenocarcinoma that have mutations in the tumor suppressor gene products p53, pRB, KAI1, and PTEN. IFN alpha (IFNα) was found to inhibit cell replication and colony formation of these cells. Analysis by flow cytometry suggests that IFNα inhibited the progression of DU145 cells from the G1 through S phase of the cell cycle. IFNα treatment of DU145 cells reduced cyclin dependent kinase 2 (cdk2) activity. In particular, cyclin E dependent cdk2 activity was inhibited by IFNα treatment. Consistent with these data, IFNα was able to induce expression of the kinase inhibitor p21 in

DU145 cells. These data support a role for p21 in mediating the antiproliferative action of IFNα and describe a mechanism for IFN action.

I additionally examined the role of IFNγ on the cell cycle of DU145 cells. IFNγ was able to inhibit DU145 cell proliferation using a similar mechanism of p21 induction. This induction of p21 correlated to an increase in p21 bound cdk2 and PCNA. Interestingly, while both IFNα and IFNγ were found to inhibit the DU145 cell cycle, only IFNγ was able to induce phenotypic changes in these cells that resulted in an antitumor effect. IFNγ treated cells exhibited a change in cellular morphology when compared with IFNα and untreated cells. These changes in morphology corresponded to a change in several cell surface receptors such as the EGF receptor. Most significantly, these phenotypic changes correlated with a decrease in the metastatic potential of DU145 cells. These results suggest that IFNγ is a superior antitumor agent to IFNα for DU145 cells. The overall implications of these findings describe a mechanism for the antitumor activities of both type I and type II IFNs in a prostatic adenocarcinoma which has mutations in genes closely involved in cell cycle control and cell adhesion.

CHAPTER 1
INTRODUCTION

Discovery of Interferons

Interferons (IFNs) were first discovered in 1957 by Isaacs and Lindenmann. They found that the treatment of chick chorio-allantoic membrane fragments with heat inactivated influenza virus resulted in the interference of the ability of fresh influenza virus to replicate in these tissues (Isaacs and Lindenmann, 1957). These observations led to the identification of a soluble factor produced in response to a viral challenge which they termed "interferon". The long term results of these experiments have been the discovery of several proteins which fall under the category of IFNs. These proteins have been found in all higher vertebrates including humans and have molecular weights ranging from 15 to 30 KD (Gastl and Huber, 1988). The human IFN proteins have been characterized and their genes cloned and expressed (Gray and Goeddel, 1982; Henco et al., 1985).

IFNs are a family of glycoproteins that are distinguishable based on their cellular source, immunological reactivity, and induction of biological responses. Original nomenclature identified the IFNs based on the cellular sources by which they are produced as well as their antibody reactivity. The current system of IFN nomenclature is based on the naming convention agreed upon in 1980 whereby IFNs were named using Greek letters. This

Table I: An overview of the interferons[a]

Type	Member	Main cellular source
I	α & ω	leukocytes[b]
	β	fibroblasts[b]
	τ	trophoblasts
II	γ	lymphocytes

[a]reviewed in Baron et al., 1991
[b]other cellular sources include epithelial cells, macrophages, and virally infected cells

system distinguishes two main types of IFN, type I and type II. Type I IFNs include alpha (α), beta (β), omega (ω), and tau (τ). Type II IFNs include only one member, IFN gamma (γ). An overview of the different IFNs as well as their cellular sources is presented in Table 1. Currently, in humans, more than 18 IFNα genes and pseudogenes have been described while only a single gene has been found for either IFNβ or IFNγ (Sen and Lengyel, 1992). Several genes have been found for IFNω and IFNτ (Bazer and Johnson, 1991; Sen and Lengyel, 1992).

Biological Activities of IFNs

IFNs are cytokines that are produced and secreted by a variety of cell types in response to several classes of inducers (Gastl and Huber, 1988). They trigger a multitude of cellular responses including antiviral actions, inhibition of cell growth and proliferation, regulation of the expression of specific genes, modulation of cell differentiation, and immunoregulation (Gastl and Huber, 1988). The specific effects of IFNs are dependent upon both the type of IFN and the target cell. A review of the current information concerning the different IFN functions is discussed below. New studies into complex network of IFN effects are constantly being carried out and the discovery of additional IFN regulated genes is ongoing. Table 2 lists the general biological activities of the different types of IFN.

IFNs were originally discovered as a result of their potent antiviral activity, and thus, this action of IFNs is perhaps the best understood. Recent

Table II: General biological activities of IFNs[a]

Antiviral
Antiproliferative
Regulation of cell growth
Immunoregulatory activities
Modulation of cell differentiation
Regulation of oncogene expression
Regulation of specific genes

[a]reviewed in Baron et al., 1991; Sen and Lengyel, 1992

experiments using gene knockout mice illustrate the critical role that IFNs play in the defense against viral infections (Huang et al., 1993). The double-stranded RNA synthesized as an intermediate in the replication of many DNA and RNA viruses triggers IFN production in cells which is released into the surrounding environment (Lengyel, 1982). The binding of released IFN by specific receptors on neighboring cells protects these cells from viral infection. Several general themes have emerged as to how IFNs are responsible for cellular protection from viral infection. The IFN system can impair various steps of viral replication, including penetration, uncoating, transcription, translation, and the assembly of progeny viruses (Lengyel, 1982; Petska et al., 1987; Samuel, 1988; Staeheli, 1990). Among the antiviral IFN inducible genes are two enzymes that inhibit viral protein synthesis: P1/eIF-2 protein kinase (Samuel, 1979), and a 2'-5' oligoadenylate synthetase (2-5AS) (Kerr, 1987). The 2-5AS enzymatically degrades viral RNA, reducing its translation into viral proteins. The eIF-2 protein kinase reduces the translation of viral proteins by decreasing the efficiency of the initiation of protein synthesis. IFNs also induce the expression of the Mx family of proteins (Staeheli et al., 1986). Induction of these proteins blocks replication of influenza virus in cultured cells and in mice possibly by blocking viral transcription (Staeheli, 1990). Some of the other antiviral effects of IFNs are mediated through the activation of different aspects of the immune system, as will be discussed later.

Soon after the characterization of IFNs as antiviral agents, it was observed that, following exposure to IFN, the replication of some cell types was inhibited (Pauker et al., 1962). IFNs are now known to exert antiproliferative effects on a variety of cell types including normal cells, immortalized cell lines, and tumor cells of various histological origins (Gastl and Huber, 1988). Much of the current IFN research centers around the complex system of IFN actions on the various proteins involved with cell growth. IFNs inhibit cell replication by lengthening the time required for progression of IFN treated cells through the cell cycle (Fleischman and Fleischman, 1992). This was later found to be a result of IFNs affecting the G1 and/or S phases of the cell cycle (Creasey et al., 1980; Pontzer et al., 1991; Roos et al., 1984; Tamm et al., 1987). IFNs exert these inhibitory activities by acting on multiple cellular pathways. Several key cell cycle proteins are affected by IFNs including phosphorylation state of the tumor suppressor gene product retinoblastoma protein (pRB) and the expression of the proto-oncogene c-myc (Einat et al., 1985a; Einat et al., 1985b; Jonak and Knight, 1984; Melamed et al., 1993). IFNs may also inhibit cell replication by depleting cells of essential metabolites. IFNs block the induction of the enzyme ornithine decarboxylase (Sekar et al., 1983) and induce indoleamine 2,3 dioxygenase (Yasui et al., 1986). A reduction in ornithine decarboxylase synthesis decreases the biosynthesis of putrescine and other essential polyamines. Indoleamine 2,3 dioxygenase causes the degradation of the essential amino acid tryptophan. Although progress in the area of IFN antiproliferative actions has been made, the

mechanisms behind these effects, especially with respect to the cell cycle, remain to be elucidated.

In addition to antiviral and antiproliferative activities, IFNs are also important immunomodulatory cytokines and they exert numerous immunoregulatory effects. IFNs upregulate the surface expression of the major histocompatibility complex (MHC) class I and class II antigens on a variety of cell types (Sen and Lengyel, 1992). MHC class I molecules are required for cytotoxic T lymphocyte (CTL) activity and MHC class II molecules are necessary for antigen presentation to helper T cells. IFNs can also increase interleukin-2 (IL-2) receptor expression (Johnson and Farrar, 1983). A number of immune cells are activated by IFNs. IFNs enhance CTL activity (Chen et al., 1986; Herberman, 1986), activation of NK cells (Giedlund et al., 1987; Weigent et al., 1983; Tuo et al., 1993), and activation of macrophage phagocytosis (Baron et al., 1991). These cytokines induce resting CTL cells to an activated state and also directly induce NK cells to exhibit enhanced cytotoxic function. Numerous macrophage functions including tumor cell cytotoxicity, antimicrobial activity, increase in killing of intracellular pathogens, and antigen processing and presentation are activated by IFNs (Degre and Bukholm, 1988; Black et al., 1988; Nathan et al., 1983; Neisel et al., 1986). They also affect the production of antibodies by B cells and can regulate the isotypes of the immunoglobulins secreted during the humoral immune response (Finkelman et al., 1988; Johnson and Torres, 1983; Snapper et al.,

Table III: Interferon inducible proteins[a]

Designation	Characteristics	Inducer
(2′-5′)$(A)_n$synthetase	(2′-5′)$(A)_n$synthesis	$\alpha, \beta > \gamma$
p68 kinase	protein phosphorylation	$\alpha, \beta > \gamma$
Indoleamine 2,3-dioxygenase	tryptophan degradation	$\gamma > \alpha, \beta$
P56	trp-tRNA synthetase	$\gamma > \alpha, \beta$
GBP/γ67	guanylate binding	$\gamma > \alpha, \beta$
Mx families	anti-influenza virus	$\alpha, \beta > \gamma$
IRF1/ISGF2	transcription factor	α, β, γ
IRF2	transcription factor	α, β
MHC class I	immune system	α, β, γ
MHC class II	immune system	γ
β_2-microglobulin	immune system	α, β, γ
IP10	platelet factor 4 related	$\gamma > \alpha, \beta$
200 family	cluster of 6 genes	α, β
6-16	unknown	$\alpha, \beta > \gamma$
1-8/9-27	unknown	α, β, γ
C56, 561	unknown	$\alpha, \beta > \gamma$
ISG54	unknown	$\alpha, \beta > \gamma$
ISG15	unknown	$\alpha, \beta > \gamma$

[a]reviewed in Sen and Lengyel, 1992

1988). In summary, IFNs play an important role in the network of immune interactions that bring about and regulate immunoreactivity and the local inflammatory response.

Other properties of IFNs include regulation of cellular differentiation and antimicrobial and antiparasitic effects. As stated previously, there are two types of IFNs classes- type I and type II. Although IFNs in both these classes have similar biological functions and overlap in their effects on different cell types, there are some differences. Table 3 is a partial list of IFN-inducible proteins and which IFNs- IFNα, IFNβ, and/or IFNγ- are the inducers for each. In general, IFNγ appears to have the dominant immunoregulatory role while IFNα and IFNβ tend to be stronger inducers of antiviral proteins. One major difference is the ability of IFNγ, and not the type I IFNs, to upregulate MHC class II (Houghton et al., 1984; Schwartz et al., 1985). Both type I and type II IFNs have potent antiproliferative effects.

Interferons and Disease

The various biological properties of both type I and type II IFNs make them potential therapies for a variety of medical disorders having viral, malignant, and immune etiologies. IFNs have been studied for their therapeutic efficacy in a number of conditions, and clinical investigations into a number of diseases are ongoing (Baron et al., 1991; Johnson et al., 1994; Stuart-Harris et al., 1992). As a result, IFNs have been approved by the Food and Drug Administration (FDA) for the treatment of several diseases (Baron

Table IV: IFNs in disease therapy[a]

	FDA approved	currently in clinical trials
IFNα	chronic hepatitis B and C	throat warts caused by papillomavirus
	hairy-cell leukemia	chronic myelogenous leukemia
	kaposi's sarcoma	colon tumors
	genital warts caused by papillomavirus	kidney tumors
		bladder cancer
		malignant melanoma
IFNβ	relapsing/remitting multiple sclerosis	basal cell carcinoma
IFNγ	chronic granulomatous disease	kidney tumors
		leishmaniasis
		chronic lymphocytic leukemia
		Hodgkin's disease

[a]reviewed in Dorr, 1993; Johnson et al., 1994

et al., 1991; Dorr, 1993; Johnson et al., 1994). For example, due to potent antiviral properties, IFNα is approved for the treatment of chronic hepatitis type B and C (Dorr, 1993). The immunomodulatory functions of IFNβ has resulted in its approval for the treatment of one type of the autoimmune disease multiple sclerosis (MS) (Johnson et al., 1994). Other diseases for which IFN therapy is currently approved by the FDA include hairy cell leukemia, Kaposi's sarcoma in the acquired immunodeficiency syndrome (AIDS), and chronic granulomatous disease. Table 4 lists the current FDA approved IFN therapies as well as others which are currently in clinical trials.

The antiproliferative and immunoregulatory actions of IFNs combined with their ability to regulate proto-oncogenes and tumor supressor gene products make them particularly attractive candidates for the treatment of various cancers. The immunoregulatory roles of IFNs that result in antitumor effects include enhancement of tumor cytotoxicity by macrophages, natural killer cells, and T lymphocytes (Baron et al., 1991). In addition, IFN enhanced expression of MHC antigens and tumor specific antigens result in more efficient recognition and killing of tumor cells by cytotoxic leukocytes (Baron et al., 1991). The induction of antibodies to the tumor cells may also be enhanced by IFNs (Baron et al., 1991). The antiproliferative capabilities of IFNs can directly inhibit replication of cells to decrease the growth rate of tumors and malignant cells (Fleischman and Fleischman, 1992). Furthermore, the ability of IFNs to modulate the expression of tumor supressor genes and proto-oncogenes has strong implications concerning

their antiproliferative capabilities. The proto-oncogene c-myc has been found to be overexpressed in a large number of cancers (Steiner et al., 1996). IFNs capability to downregulate c-myc expression could contribute significantly to control tumor growth, especially in tumors where c-myc overexpression is involved in the malignancy. IFNs express potent antitumor effects both by exerting direct antiproliferative effects on target tumor cells, through the enhancement of immune responses, and by activating host cytotoxic effector cells to more efficiently lyse target tumor cells.

One obstacle facing the widespread clinical use of IFNs is the toxic side effects experienced by some populations of patients (Vial and Descotes, 1994). Adverse effects associated with IFNs are usually acute effects that involve "flu-like" symptoms that include fever, malaise, tachycardia, chills, headache, arthralgias, and myalgias (Gauci, 1987; Quesada et al., 1986; Spiegal, 1987). However, these symptoms are usually not treatment limiting and are tolerable using symptomatic treatment (Vial and Descotes, 1994). The symptoms are also reversible after reduction in IFN dosage. A recently discovered type I IFN, IFNτ, provides a non-toxic alternative. IFNτ was originally recognized as a pregnancy recognition hormone in ruminants (Bazer and Johnson, 1991). It has now been shown to have similar biological activities to the other type I IFNs but without the associated toxicity (Pontzer et al., 1988; Pontzer et al., 1991, Soos and Johnson, 1995a; Soos et al., 1995b). IFNτ has been shown to block experimental allergic encephalomyelitis (EAE)

in mice and may provide an alternative therapy to IFNβ for the treatment of MS (Soos et al., 1995b).

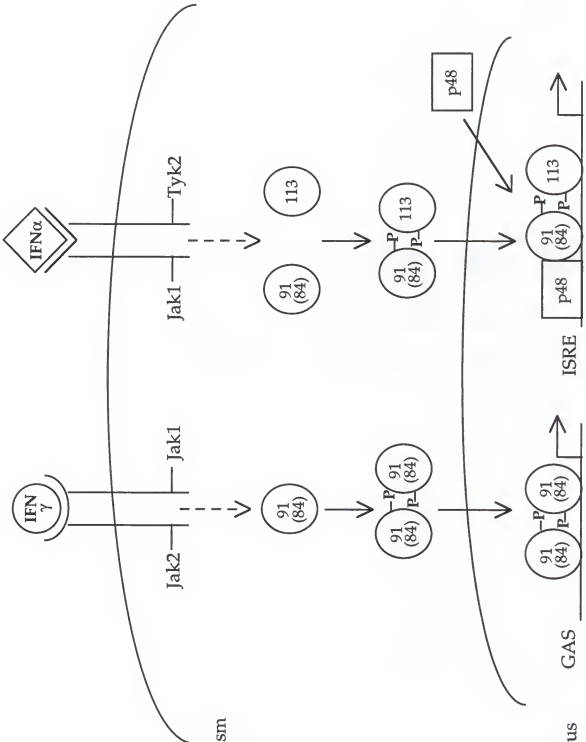
Interferons and Signal Transduction

The binding of IFNs to their specific receptor is the first step in evoking their biological responses. All type I IFNs bind to the same cell surface receptor while IFNγ binds to a separate, although similar, receptor (Branca, 1988). The binding of type I IFNs to their receptor brings together two receptor chains, the IFNα/β receptor 1 chain and IFNα/β receptor 2.2 chain (Domanski et al., 1995; Novick et al., 1994; Lutfalla et al., 1996; Uze et al., 1990). These chains are induced to associate in the presence of ligand resulting in the formation of a functionally active receptor which mediates type I IFN signaling (Cohen et al., 1995; Darnell et al., 1994). Additionally, a third component of the type I receptor is believed to exist due to the differential effects of the type I IFNs (Croze et al., 1996). A receptor complex consisting of several subunits may explain how the different type I IFNs elicit the preferential induction of IFN specific genes while still binding the same receptor (Petska et al., 1987; Rani et al., 1996). The IFNγ receptor consists of two integral membrane polypeptides that include α and β subunits (Aguet et al., 1988; Farrar and Schreiber, 1993; Soh et al., 1994). The α subunit is necessary for ligand binding while the β subunit participates in signal transduction (Farrar and Schreiber, 1993). IFNγ binds its receptor as a homodimeric ligand which results in rapid dimerization of the receptor and

subsequent signal transduction (Fountoulakis et al., 1992; Greenlund et al., 1993). As a result of ligand binding with both the type I and type II receptors, signal transduction is surprisingly rapid. Within fifteen minutes, transcription of chromosomal genes is enhanced without the need for new protein synthesis (Friedman et al., 1984; Larner et al., 1984; Larner et al., 1986; Levy and Darnell, 1990; Reich et al., 1987). This rapid transmission of signals combined with the apparant lack of involvement of second messengers has created an area of intense research in recent years concerning the signal transduction of the IFNs.

The signal transduction pathway of the IFNs is direct in nature. Stimulation of the type I and type II IFN receptors initiate the activation of a class of tyrosine kinases known as Janus kinases or JAKs (Darnell et al., 1994). Type I IFNs activate two tyrosine kinase called tyrosine kinase 2 (tyk2) and JAK1 (Silvennoinen et al., 1993; Velazquez et al., 1992). These activated kinases subsequently phosphorylate three proteins known as STAT113, STAT91, and STAT84 (Fu et al., 1992; Silvennoinen et al., 1993). The term "STAT" stands for signal transducer and activator of transcription while the number designates the molecular weight of the protein. The phosphorylation of the STATs causes them to associate with one another to form a complex which, combined with another protein referred to as p48, acts as a transcription complex to directly control gene transcription (Fu et al., 1990; Silvennoinen et al., 1993). The type I IFN STAT complex binds a target sequence motif known as the interferon-stimulated response element (ISRE)

Figure 1. The IFN signal transduction pathways. IFNs bind their receptors and initiate intracellular signaling events that involve the JAK/STAT system. IFNγ binds as a homodimer and causes the phosphorylation of JAK1 and JAK2. JAK1 then phosphorylates a latent cytoplasmic transcription factor STAT91. Phosphorylated STAT91 dimerizes and translocates to the nucleus where it binds the specific promoter elements known as GAS. This binding leads to the transcription of IFNγ inducible genes. A similar mechanism is used by type I IFNs but utilizes JAK1 and TYK2 which phosphorylate STAT84/91 and STAT113. These STATs combine with p48 and enter the nucleus where they activate transcription of genes containing ISRE sequences in their promoter regions.


IFN γ
Jak2
Jak1
IFNα
Jak1
Tyk2
cytoplasm
91 (84)
113
P
nucleus
GAS
ISRE
p48

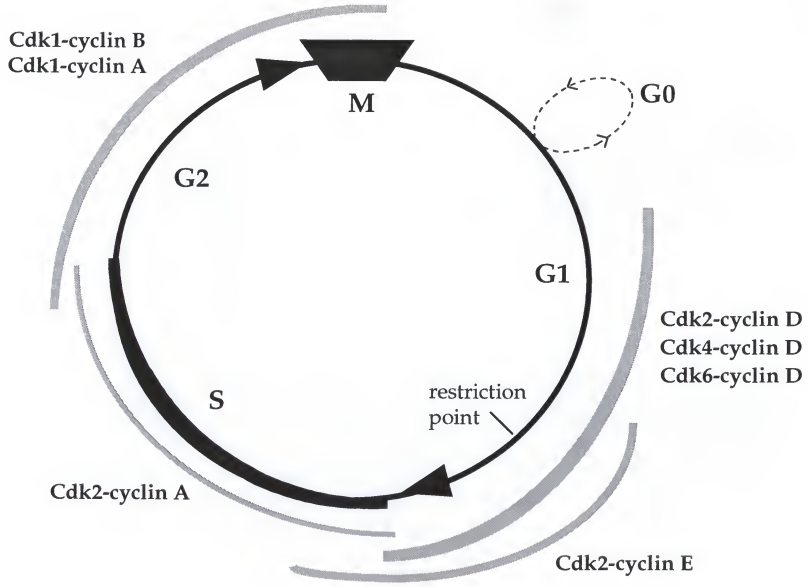
(Kessler et al., 1990). The IFNγ activation pathway is similar in that it utilizes two JAKs, one of which is shared by the type I system. JAK1 and JAK2 phosphorylate STAT91 which forms a homodimer and acts as a transcription factor which binds to specific promoter elements designated as gamma activation sites (GASs) (Decker et al., 1991; Greenlund et al., 1994; Igarashi et al., 1994; Lew et al., 1991; Shuai et al., 1994). JAK/STAT systems similar to those used by IFNs have been found to be utilized by a large array of cytokines (Leaman et al., 1996). An overview of the IFN signal transduction pathways is presented in Figure 1.

The Mammalian Cell Cycle

As previously mentioned, IFNs are believed to exert their antiproliferative effects by inhibiting the progression of the cell cycle. This may be due in part through the modulation of proteins involved in cell cycle regulation. The mechanism behind the anticellular effects of IFNs is poorly understood. The regulation of the cell cycle is particularly relevant in the study of IFNs in cancer therapy.

The mammalian cell cycle consists of five phases: G0, G1, S, G2, and M (Figure 2) (reviewed in Grana and Reddy, 1995). The G0 represents the quiescent phase where cells are considered to "exit" the cycle and remain in a non-replicating state. Once the cell is stimulated by growth factors or mitogens to re-enter the cell cycle, it is in the G1 phase where it prepares for DNA replication. In the S phase, cellular DNA is replicated. The G2 phase

Figure 2. The mammalian cell cycle. The cell cycle consists of five phases G0, G1, S, G2, and M. The cdk-cyclin complexes are outlined and listed next to each cell cycle phase.


Cdk1-cyclin B
Cdk1-cyclin A
M
G0
G2
G1
Cdk2-cyclin D
Cdk4-cyclin D
Cdk6-cyclin D
S
restriction
point
Cdk2-cyclin A
Cdk2-cyclin E

prepares the cell for mitosis, which is followed by the M phase where the cell physically divides. A multitude of cell proteins and biochemical pathways coordinate the cell cycle and the regulation of the system is complex. The following provides a condensed overview of the key proteins involved in the mammalian cell cycle.

The progression of the cell cycle is directly dependent upon the activity of a set of protein kinases known as the cyclin dependent kinases (cdks). As the name suggests, cdks are kinases which require regulatory subunits called cyclins in order to be active (Morgan, 1995). Cyclins are named for their cyclic nature of expression. They are specifically expressed during each phase of the cell cycle and are promptly degraded as the cycle advances (reviewed in Koff et al., 1992; Sherr, 1994). Figure 2 outlines the cdk-cyclin complexes which are active during each cell cycle phase. Briefly, cdk4/cdk6 binds to cyclin D in G1, cdk2 to cyclin E in G1 to S phase transition, cdk2 to cyclin A in S phase, and cdk1 (cdc2) to cyclin A/B in M (Koff et al., 1992; Nurse, 1990; Sherr, 1993). These cdk-cyclin partners phosphorylate proteins which cause a cascade of events resulting in the coordination of cell cycle progression. Cell cycle control is carried out in large part by regulating cdk activity using a variety of mechanisms and feedback loops.

One group of proteins that is directly involved in the regulation of cdk activity are the cyclin dependent kinase inhibitors (CKIs) (Elledge and Harper, 1994). These inhibitors are induced in response to specific extracellular signals and play an important role, via the inhibition of cdk activity, in

Table V: Cyclin-dependent kinase inhibitors[a]

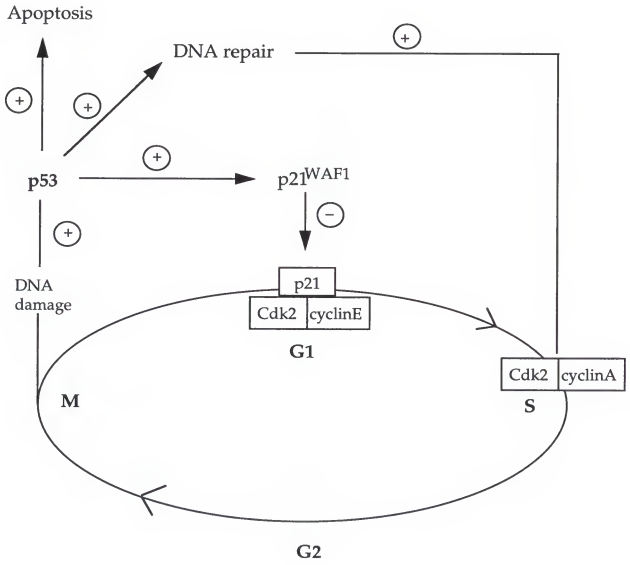
Cell cycle phase	Cyclin-cdk complexes	Inhibitors					
		p15	p16	p18	p19	p21	p27
G1	Cdk4/6-cyclin D	+	+	+	+	+	+/-
G1/S	Cdk2-cyclin E	--	--	--	--	+	+
S	Cdk2-cyclin A	--	--	--	--	+	--
G2/M	Cdk1-cyclin B	--	--	--	--	+	--

[a]reviewed in Grana and Reddy, 1995.

blocking the cell cycle (Elledge and Harper, 1994; Pines, 1994). CKIs inhibit cdk function by physically binding cdk-cyclin complexes and interfering with kinase activity. Table 5 lists the known CKIs and the cdk-cyclin complexes they block. Currently CKIs are separated into two families. The first includes $p21^{WAF1}$ and $p27^{KIP1}$ which share partial identity and are involved primarily with blocking G1 and S phases of the cell cycle (Harper et al., 1993; Toyoshima and Hunter, 1994; Xiong et al., 1993). Both $p21^{WAF1}$ and $p27^{KIP1}$ are present in quiescent cells and can cause G1 arrest. $p21^{WAF1}$ was the first CKI to be identified and is considered to be a universal CKI (El-Deiry et al., 1993; Gu et al., 1993). $P27^{KIP1}$ has been found to be induced in response to TGFβ (Polyak et al., 1994). The other family of CKIs is the INK family which includes p15, p16, p18 and p19 (Guan et al., 1994; Hannon and Beach, 1994; Hirai et al., 1995; Serrano et al., 1993). These CKIs are important for blocking cdk4/cdk6 activity in early G1 phase and can also cause G1 arrest (Hirai et al., 1995; Serrano et al., 1993). Interestingly, p16 has been designated a tumor suppressor gene since it has been found that mice with deletions in the p16 gene have a dramatic increase in tumor formation (Serrano et al., 1996). Other CKIs are potential candidates for tumor suppressor genes due to their regulatory impact on the cell cycle.

The prototypic tumor suppressor gene product p53 is a transcription factor that has multiple roles in cell cycle regulation, and for this reason is one of the most commonly mutated proteins found in malignant cells (reviewed in Levine et al., 1991). Many of the functions of p53 in cell

Figure 3. The role of p53 in the G1 checkpoint. DNA damage induces p53 expression. p53 can then lead to the induction of $p21^{WAF1}$, apoptosis, or facilitate DNA repair. The induction of $p21^{WAF1}$ results in the inhibition of cdk-cyclin complexes including cdk2-cyclin E. This delay in the progression of the cell cycle allows time for DNA repair before DNA replication in the S phase.


Apoptosis
DNA repair
+
+
+
+
p53
p21WAF1
+
−
DNA
damage
p21
Cdk2
cyclinE
G1
Cdk2
cyclinA
S
M
G2

replication have been determined, one of which is its role in cell cycle checkpoints (Kastan et al., 1992, Kuerbitz et al., 1992). Cell cycle checkpoints represent the coordination of the cell cycle machinery with the biochemical pathways that respond to DNA damage and restore its structure (Kaufman et al., 1995). Checkpoints at G1 and G2 phases delay the cell cycle and provide more time for repair before the critical phases of DNA replication and mitosis. DNA damage in cells induces p53 expression which causes G1 arrest (Kuerbitz et al., 1992). It has been found that this arrest is, at least in part, due to p53 induction of the CKI $p21^{WAF1}$ (El-Deiry et al., 1993; El-Deiry et al., 1994). $p21^{WAF1}$ blocks cdk2-cyclin E activity and stops the cell at the G1 checkpoint (Xiong et al., 1993). Another function of p21 is to bind the proliferating cell nuclear antigen (PCNA) and block DNA replication (Flores-Rozas et al., 1994; Waga et al., 1994). PCNA is an essential component of the DNA replication machinery (Kelman, 1997). p53 is also believed to play a role in the G2 checkpoint and mitosis (Guillouf et al., 1995). However, the mechanism behind this checkpoint is not fully understood. p53 has other roles in the cell, including involvement in apoptosis. An overview is provided in Figure 3.

pRB and c-myc are two other proteins that are commonly found to be abnormally expressed in malignant cells (reviewed in Levine, 1993; Marcu et al., 1992). They have also been found to be modulated by IFNs. pRB is a tumor supressor that acts as the main target for G1 and S phase cdk-cyclin phosphorylation (Cobrinik et al., 1992). The G1 cdk-cyclin complexes phosphorylate pRB to a hyperphosphorylated state (Grana and Reddy, 1995).

In this state, pRB is unable to bind the E2F transcription factor. E2F is then free to transcribe a number of genes that are involved in cell replication. C-myc is an oncogene whose overexpression leads to uncontrolled cell growth (Marcu et al., 1992). It binds with its partner, max, to form a heterodimeric transcription factor (Amati et al., 1993). This dimer transcribes a number of genes that promote cell growth. By blocking c-myc expression and the phosphorylation of pRB, IFNs can regulate the transcription of proteins that promote cell replication.

Adhesion Molecules and Growth Factor Receptors

Adhesion molecules encompass another area of intense research due to their significance in tissue development, tumor development, and the immune response. These surface receptors play critical roles in cell-cell and cell-extracellular matrix (ECM) interactions. Pathologic alterations in these adhesion properties underlie many of the phenotypic changes associated with tumor progression, including changes in cell morphology, migration, tissue invasiveness, and metastatic potential (reviewed in Hannigan and Dedhar, 1997). There are several classes of cellular adhesion molecules (CAMs): integrins, cadherins, selectins, and the immunoglobulin superfamily. The integrins comprise a family of widely expressed transmembrane receptors that are expressed on all cell types and mediate cell-cell and cell-ECM interactions (Hynes, 1992). They are named according to the combination of different α and β subunits which form a heterodimeric receptor. This pairing of different

subunits provides receptor diversity. The expression of certain integrin subunits is tissue or cell specific, such as with the integrin $\alpha_L\beta_2$ (LFA-1) whose expression is limited to leukocytes (Stewart et al., 1995). The members of the immunoglobulin superfamily contain typical immunoglobulin-like domains in the extracellular portion of the molecule and are expressed on a variety of cell types (Hannigan and Dedhar, 1997). They play an important role in the inflammatory immune response and modulate tumor spread by regulating the interaction of circulating tumor cells with host immunocytes. For example, the intercellular adhesion molecule-1 (ICAM-1) is a member that can bind the integrin receptor LFA-1 (Marlin and Springer, 1987). LFA-1 is found on leukocytes including NK cells and CTLs (Makgoba et al., 1988; Dana and Arnaout, 1994). ICAM-1 and LFA-1 binding can form stable cytolytic conjugates between tumor cells and cells of the immune system (Hannigan and Dedhar, 1997). The selectins and cadherins are involved with cell-cell interactions via carbohydrate moieties and homophilic binding, respectively (Hart, 1996).

Growth factors can influence constitutive activation of growth promoting pathways in cancer cells and can modulate cell phenotype (reviewed in Aaronson, 1991). A large array of factors have been discovered that affect the growth of virtually all cell types which can act as positive or negative modulators of cell proliferation and influence differentiation. Many growth factors cause cells in the G0 phase to re-enter the cell cycle (Pledger et al., 1977). For this reason, several oncogenes encode growth factors and

tyrosine kinase receptors that participate in mitogenic signaling (Bishop, 1991). There is much evidence for genetic aberrations affecting growth factors and their receptors in human malignancies. Among growth factor receptors, the most frequently implicated in human cancer are the members of the EGF receptor (EGFR) family (Aaronson, 1991). The EGFR is a tyrosine kinase receptor that binds both EGF and TGFα ligands. The EGFR gene is often amplified or overexpressed in squamous cell carcinomas and glioblastomas (Libermann et al., 1985; Yamamoto et al., 1986), and EGFR expression has been linked to poor patient prognosis in other malignancies (Kristensen et al., 1996; Nakopoulou et al., 1995). Control of the overexpression of growth factor receptors and their ligands has several implications for cancer intervention. One is the potential improvement in diagnosis and prognosis of cancer. Possibilities for cancer therapy include effective means for targeting tumor cells by blocking signal transduction or ligand function.

Experimental Rationale

IFNs are a group of proteins that trigger a multitude of cellular responses including inhibition of cell growth, modulation of cell differentiation, and immunoregulation (Gastl and Huber, 1988). For this reason, many studies have looked at the potential antitumor effects of IFNs using both in vitro and in vivo models of cancer. These studies have resulted in the use of IFNs in the treatment of several cancers such as hairy cell leukemia, CML, and kaposi's sarcoma (Dorr, 1993; Gutterman, 1994).

However, IFNs have not been successful in treating some other types of malignancies. The lack of patient improvement seen in some clinical trials may be attributed to inadequate knowledge of the underlying antitumor effects of IFNs. Thus, elucidation of the antitumor mechanisms of IFNs against a particular type of cancer cell are important for indicating which cancers may be susceptible to IFN therapy. The work included in this dissertation takes a fundamental look at the antitumor effects of type I and type II IFNs using an in vitro system involving a human prostate adenocarcinoma cell line, DU145.

Prostate cancer is the most commonly diagnosed malignancy in men (Parker et al., 1996). Like other types of cancers, prostate cancer results from a loss or mutation of regulatory factors of the cell cycle such as oncogenes and tumor suppressor genes (Cavenee and White, 1995; Garnick, 1994). Previous studies have shown that human prostate cancer cell lines are sensitive to the antiproliferative properties of IFNs (Nakajima et al., 1994; Sica et al., 1989). Although these effects have been recognized, the mechanism behind this cellular inhibition remains unclear.

DU145 is an interesting cell line to study the antitumor effects of IFNs for a number of reasons. This cell line was established from a metastatic lesion in a patient with advanced prostate cancer (Stone et al., 1978). In addition, DU145 cells have characteristics associated with undifferentiated malignant prostate cells. For example, these cells are androgen independent (Stone et al., 1978), tumorigenic in nude mice (Bookstein et al., 1990), and

have mutations in several tumor suppressor gene products including p53, pRB, PTEN, and KAI1 (Bookstein et al., 1990; Dong et al., 1995; Isaacs et al., 1991; Li et al., 1997). Examination of the antitumor effects of IFNs on DU145 cells describes a potential mechanism for their regulatory capabilities on an adenocarcinoma that has mutations in proteins closely linked to the cell cycle and cell adhesion. Studies into the mechanisms of the antitumor effects of IFNs give insight into the use of IFNs in cancer therapy. In addition, this information may indicate which cytotoxic agents and cytokines may produce synergistic combinations with IFNs and therefore provide successful clinical therapies.

CHAPTER 2
MATERIALS AND METHODS

Reagents and Cell Lines

Purified human IFNα (specific activity 2×10^8 units/ml) was obtained from Biosource International (Camarillo, CA). Purified human IFNγ (specific activity 4.75×10^7 units/mg) was obtained from Genzyme Diagnostics (Cambridge, MA). WISH and DU145 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). Complete media for DU145 cells consisted of Eagles minimal essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS), 200 U/ml penicillin, and 200 μg streptomycin. Starvation medium for cell synchronization contained the ingredients listed above supplemented with 0.5% FBS. Antibodies to cdk2, cyclin E, cyclin D, PCNA, p21, p27, and p16 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to ICAM-1 and EGF receptor were obtained from Pharmingen (San Diego, CA) for flow cytometric analysis and from Transduction Laboratories (Lexington, KY) for immunoblotting. Antibodies to integrin α3 were obtained from Oncogene Research Products (Cambridge, MA).

Antiviral Assay

IFN activity is expressed in terms of antiviral units/ml as assessed in a standard cytopathic effect assay (Familletti et al., 1981). Antiviral activity of human IFNα was determined using the WISH cell line and vesicular stomatitis virus (VSV). One antiviral unit caused a 50% reduction in destruction of the monolayer.

Antiproliferative Assays

For colony inhibition studies, anticellular activity was examined using a modification of a colony inhibition assay (Blalock et al., 1980). DU145 cells were plated at 600 cells/well in a 24 well plate using complete medium with or without various concentrations of IFNα or IFNγ. Plates were incubated at 37°C for 5-6 days to allow for colony formation. Colonies were stained with crystal violet and counted.

IFN inhibition of cell number was determined by using DU145 cells plated in complete medium at 1×10^5 cells/well in 6 well plates with or without IFN. At various time points, cells were removed from flask using 0.25% trypsin-EDTA solution (Sigma Co., St. Louis, MO) washed 2 times with phosphate buffered saline (PBS), and counted. Cell counts were performed using a hemocytometer, and cell viability was assessed by trypan blue dye exclusion (Blalock et al., 1980).

DNA Synthesis Assay

DU145 cells were seeded at 2 x 10^5 cells/well in 6 well plates using starvation medium for 24 hours. Wells were then washed and replaced with complete medium alone or medium containing 2500 units/ml IFNα. At 16, 20, and 24 hours, cells were harvested and counted as described above. Cells were then reseeded into 96 well plates at 2.5 x 10^4 cells/well and pulsed with 1 μC [^{3}H]thymidine (specific activity, 21 mCi/mg; 1 Ci= 37 Gbq) (Amersham) for 2 hours at 37°C. Cells were then harvested on a model M12 Brandel cell harvester (Gaithersburg, MD) and incorporation of [^{3}H]thymidine was determined using a liquid scintillation counter.

Cell Cycle Analysis

DU145 cells were synchronized in G0/G1 phase (sychronization was assessed by flow cytometry analysis by determining the percentage of cells in the G0 phase of the cell cycle) by culturing in starvation medium for 24 hours. Cells were seeded into 25 cm^2 flasks (Sarstedt, Newton, NC) and grown in the presence of medium alone or medium containing either IFNα or IFNγ. Plates were incubated at 37°C in 5% CO_2 for varying lengths of time. Following incubation, cells were harvested and washed 2 times using sample buffer (PBS containing 1% glucose) and were then counted. Following centrifugation, cell pellets were resuspended in 100 μl sample buffer and cells were fixed by adding 1 ml cold ethanol (95%) (-20 °C) dropwise while vortexing. Cells were left in ethanol at 4°C for ≥ 24 hours. For propidium

iodide (Sigma Co., St. Louis, MO) staining, the prepared cell samples were washed 2 times with sample buffer and blotted dry. The cells were then resuspended in 600 - 800 µl sample buffer containing 50 µg/ml propidium iodide and 100 U/ml RNase A (Sigma Co.) and allowed to stain for up to 1 hour at room temperature. Samples were filtered through 44-µm nylon mesh and data from 30,000 events was acquired with a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA) using the LYSYS II software system. Analysis of the cell cycle was carried out using CellFIT software which determines the percentage of cells in each phase of the cell cycle based on cells collected using the LYSYS II software system.

Immunoprecipitation and Immunoblotting

For preparation of cell lysates, each experiment used an equal number of DU145 cells per sample in complete medium stimulated with IFNα or IFNγ were lysed at 4°C for 20 minutes in 500 µl of ice-cold lysis buffer that consisted of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 20 mM β-glyceryl phosphate, 2 mM Na_3VO_4, 2 mM dithiothreitol, leupeptin (10 µg/ml), pepstatin (10 µg/ml), aprotinin (10 µg/ml), benzamidine (5 µg/ml), 1 mM phenylmethanesulfonyl fluoride, 10% (vol/vol) glycerol, and 1% (vol/vol) Nonidet P-40. A range of 3-6 x 10^6 cells per sample were used with individual replicate experiments. Lysates were then centrifuged at 14,000 x g for 10 minutes. Equal amounts of protein (protein levels were determined using the BCA protein assay kit (Pierce,

Rockford, IL)) from cell lysates were either directly loaded onto a polyacrylamide gel (a range of 100-175 µg per sample was used with individual experiments) and electrophoresed or subsequently treated (a range of 375-500 µg/500 µl was used with individual experiments) with 1-10 µg of antibody to the protein of interest for 1 hour at 4°C. The lysate-antibody complex was allowed to bind for an additional hour using 30-40 mg of protein A sepharose. Samples were then washed three times with 1 ml lysis buffer followed by a single wash with 1 ml of 50 mM Tris, pH 6.8. Complexes were eluted in SDS-PAGE sample buffer (0.03 M Tris-HCl, pH 6.8, 10% glycerol, 2%(w/v) SDS, 5% β-mercaptoetanol, and 2.5% bromophenol blue) and electroporesed through a Tris-HCl polyacrylamide gel with 4% stacking gel. Following Western transfer, nitrocellulose membranes were probed with antibodies to the proteins of interest and developed using the ECL chemiluminescence system (Amersham). Densitometric analysis of radiographic film using IA-200 Image Analysis Software was used to determine percent difference between band intensities based on total pixel value.

In Vitro Kinase Assays

Treatment of cell lysates with antibodies to cdk2, cyclin D1, and cyclin E were performed as described above. Antibody-protein conjugates were washed 3 times with lysis buffer and twice with kinase buffer (50 mM Hepes, pH 7.5, containing 1 mM EGTA, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM β-

glyceryl phosphate, 5 μM ATP) and incubated in kinase buffer containing 5 μg histone H1, and 20 μCi of [^{32}P]-γ-ATP (specific activity 6000 Ci/mmol; 1 mCi= 37 Mbq) (Amersham) in a final volume of 30 μl at 30°C for 10 minutes. Following centrifugation, supernatant fluids (25 μl) were analyzed for histone H1 phosphorylation by a filter-binding assay using centrifugal Pierce phosphocellulose units, SpinZyme™ Format purchased from Pierce (Rockford, IL) according to the manufacturer's instructions.

Cellular Morphology

DU145 cells in complete medium were treated, with or without IFN, for 5 days in culture slides (Falcon, Becton Dickinson, Bedford, MA). Slides were washed 3 times with phosphate buffered saline (PBS), fixed, and stained with eosin-methylene blue. Slides were then analyzed by light microscopy.

Flow Cytometric Analysis of Surface Receptors

DU145 cells were treated with IFN as described above. Cells were then harvested and washed 3 times with PBS. Equal number of cells per sample (8-10 x 10^5 cells/sample) were washed 3 times with flow cytometry buffer (PBS containing 0.1% Na azide, 5% FBS) and incubated for 1 hour at 4°C with antibodies specific for EGFR (2 μg/ml), ICAM-1 (1 μg/ml), or integrin α3 (4 μg/ml). After washing, cells were incubated for an additional hour with FITC labeled secondary antibodies specific for mouse IgG1. Data from 30,000 events

were acquired as described above and analyzed using Median Fluorescence Intensity (MFI) software.

Analysis of Growth Factor Production

DU145 cells were treated as described above for 5 days and reseeded at 1 x 10^5 cells/well into 6 well plates in EMEM (no FBS). Supernatant fluids were harvested after 24 hours and used in a Cytokine Total ELISA kit (Intergen, Purchase, NY) specific for human EGF.

Invasion Chamber Migration

DU145 cells were treated as described above for 5 days and reseeded, without IFN or FBS, at 1 x 10^5 cells/well into Biocoat Matrigel invasion chambers (Falcon, Becton Dickinson, Bedford, MA). The chambers were then placed into 24 well plates containing EMEM supplemented with 20% FBS. These plates were then incubated 36-48 hours at 37°C. Membranes from each chamber was then removed, subsequently fixed, and stained with eosin-methylene blue. Total number of invasive cells was then determined.

CHAPTER 3
RESULTS

IFNα Inhibition of the DU145 Cell Cycle

Inhibition of Cell Growth

The investigation into the effects of IFNα on prostate cancer cells was started by determining the antiproliferative properties of IFNα on DU145 cells, a human prostate cancer cell line. IFNα inhibited colony formation of DU145 cells at low cell density (600 cells/well) in a dose dependent manner as shown in Table VI. The antiproliferative effects of IFNα was also determined by utilizing direct cell counts. DU145 cells were treated with 2500 units/ml of IFNα and the overall reduction in cell number was determined (Figure 4). A reduction in the rate of growth by approximately 50% was observed in IFN treated cultures versus untreated cultures. These results indicate that IFNα has antiproliferative activity on DU145 cells.

Reduction in ^{3}H-Thymidine Incorporation

In examining the effects of IFNα on the DU145 cell cycle, the incorporation of [^{3}H]thymidine by cultures synchronized, by serum starvation, into G0/G1 was first determined (Figure 5). The incorporation of thymidine by cells is a measure of chromosomal replication, and is therefore an indication of cellular activity in the S phase of the cell cycle (Tamm et al.,

Table VI: IFNα inhibition of colony formation of DU145 cells[a]

IFNα (units/ml)	colonies/ well (mean±SD)	inhibition (%)
0	77.7 ± 4.2	--
625	60.3 ± 4.7	22.4
1250	40.3 ± 3.5	48.1
2500	33.7 ± 3.2	56.6
5000	30.6 ± 2.1	60.6

[a]DU145 cells were plated at 600 cells/well with various doses (units/ml) of IFNα for 6 days and subsequently stained with crystal violet. Samples were assessed in triplicate and results are expressed as the mean number of colonies ± SD. Statistical significance was shown by Student's t-test between the number of colonies in the presence or absence of 625 U/ml ($p<0.05$), 1250 U/ml ($p<0.02$), 2500 U/ml ($p<0.006$) and 5000 U/ml ($p<0.006$) of IFNα.

Figure 4. IFNα inhibition of DU145 cellular proliferation. Synchronized DU145 cells (1 x 10^5/well) were incubated with or without 2500 units/ml IFNα for 48, 72, or 96 hours. Wells were then harvested and the total number of live cells determined. Data are expressed as total number of cells per sample ± SD for three replicates. Statistical significance was shown by Student's t-test between the number if cells in the presence and absence of 2500 U/ml IFNα for 48 ($p<0.03$), 72 ($p<0.04$) and 96 ($p<0.03$) hours.

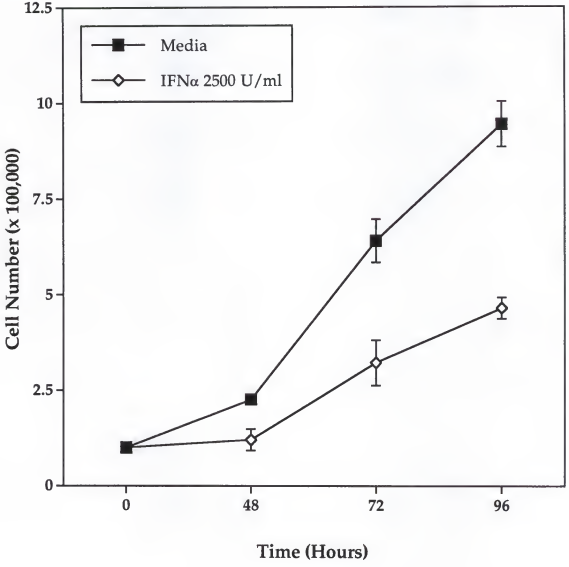
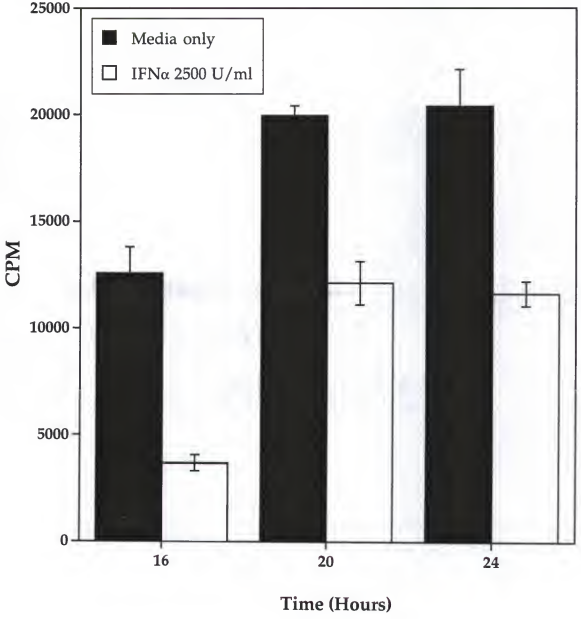

12.5
10
7.5
5
2.5
0
Cell Number (x 100,000)
Media
IFNα 2500 U/ml
0
48
72
96
Time (Hours)

Figure 5. Treatment of DU145 cells with IFNα inhibits [^{3}H]thymidine incorporation. DU145 cells (1 x 10^5) synchronized in G0/G1 were incubated with or without IFNα for 16, 20, or 24 hours. Cells were harvested and reseeded into 96 well plates at 2.5 x 10^4 cells/well, incubated with [^{3}H]-thymidine for 2 hours, and harvested on a cell harvester. Data are expressed as mean cpm ± SD for six replicates. Statistical significance was shown by Student's t-test between [^{3}H]thymidine incorporation by cells in the presence and absence of 2500 U/ml IFNα for 16 ($p<0.0006$), 20 ($p<0.0003$) and 24 ($p<0.0006$) hours.


25000
20000
15000
10000
5000
0
CPM
Media only
IFNα 2500 U/ml
16
20
24
Time (Hours)

1987). DU145 cells were incubated with or without 2500 units/ml IFNα for 16, 20, or 24 hours and pulse-labeled at each time point with [^{3}H]thymidine. At 16 hours, the amount of [^{3}H]thymidine incorporated by IFN treated cells was only 29% of the incorporation seen with the untreated cells. At 20 and 24 hours, IFN treated cells incorporated about 60% of the [^{3}H]thymidine incorporated by untreated cells. These reductions show that IFN treated cells entered the S phase at least 4 hours later than untreated cells. Consistent with this, at 20 and 24 hours, IFN treated cells reached a level of [^{3}H]thymidine incorporation seen with untreated cells at 16 hours. These data suggest that IFNα inhibited the progression of DU145 cells from G1 through S phase.

Flow Cytometric Analysis of the Cell Cycle

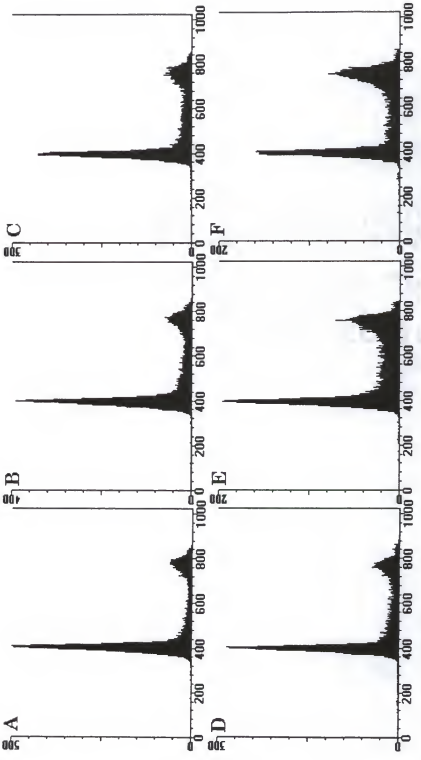
The inhibitory effects of IFNα on the DU145 cell cycle were further examined using flow cytometry analysis (Table VII, Figure 6). DU145 cells synchronized by serum starvation into G0/G1 were stimulated to enter the cell cycle by serum addition in the presence or absence of 2500 units/ml IFNα. As can be seen from Table VII, untreated cells rapidly advanced through the G0/G1 and S phases, and, by 40 hours, cells had already completed one full cycle and were again entering the S phase. IFN treated cells, however, progressed more slowly. After 24 hours, 54% of the IFN treated cells were still in the G0/G1 phase, while untreated cells had only 39% of cells in G0/G1. The flow cytometry histograms (Figure 6) depict the state of the DU145 cell

Table VII: Cell cycle analysis of IFNα treated DU145 cells[a]

Time (hours)	IFNα (units/ml)	cell cycle phase (%)		
		G0/G1	S	G2/M
0	0	63.0	18.5	18.5
16	0	54.9	33.6	11.5
	2500	66.9	22.1	11.0
20	0	39.5	46.2	14.2
	2500	57.8	30.9	11.3
24	0	39.2	35.4	25.4
	2500	53.8	33.7	12.5
40	0	62.1	24.9	13.0
	2500	48.7	32.7	18.6

[a]DU145 cells were treated with 0 or 2500 units/ml for 0, 16, 20, 24, or 40 hours. Progression through the cell cycle was examined using propidium iodide staining. Data from 30,000 events are presented as percentage of cells in each stage of the cell cycle. Similar results were seen with two replicates of this experiment.

Figure 6. IFNα inhibits the progression of DU145 cells through G1 and S phase of the cell cycle. DU145 cells were synchronized in G0/G1 by growing in medium containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide. *Horizontal axis*, relative fluorescence intensity; *vertical axis*, number of cells. *A*, *B*, and C IFNα treated cells (2500 units/ml); *D*, *E*, and *F*, untreated controls, at 16, 20, and 24 hours after the initiation of culture, respectively. Similar results were seen with two replicates of this experiment.


A
B
C
D
E
F
0
200
400
600
800
1000

cycle at 16, 20, and 24 hours in the presence and absence of IFN. A similar pattern was seen with IFN treatment at 1000 units/ml (data not shown). Consistent with the [^{3}H]thymidine incorporation by IFNα treated DU145 cells, flow cytometry analysis showed that IFNα inhibited progression of prostate cancer cells through G1 and early S phase of the cell cycle.

Inhibition of Cdk2 Activity

In order to determine the relationship of the phase of the cell cycle specifically inhibited by IFNα in the context of cyclin dependent kinase (cdk) activity, the activity of a cdk, cdk2, that is active during the G1 and S phases of the cell cycle was examined (Pines and Hunter, 1995). DU145 cells were cultured with or without IFNα for 16 or 24 hours and subsequently harvested. For each time point, cdk2 was immunoprecipitated and function assessed by histone H1-dependent kinase activity (Table VIII). DU145 cells synchronized to G0/G1 had low cdk2 activity at 16 hours. However, by 24 hours, these cells increased their cdk2 activity by greater than 10-fold, while IFN treated cells showed only a 4-fold increase, resulting in a 74% reduction of cdk2 activity over the control. The data show that IFNα is able to reduce the activity of a cdk specific for the G1 and S phases of the cell cycle in prostate cancer cells, and thus inhibit the progression through the cell cycle.

Table VIII: Effect of IFNα treatment on cdk2 activity[a]

Time (hours)	IFNα (units/ml)	CPM	reduction (%)
16	0	8042	--
	2500	6138	23.7
24	0	94880	--
	2500	24570	74.1

[a]DU145 cells were treated with 2500 units/ml IFNα for 16 or 24 hours. Cyclin dependent kinase 2 activity was assessed by histone H1-dependent kinase activity. Cdk2 activity is represented as cpm with corresponding percent reduction. Similar data with the same patterns were observed in three repeats of this experiment.

Analysis of Cyclin E and Cyclin D Dependent Cdk2 Activity

To further examine the stage of the cell cycle that is regulated by IFNα, the cyclin specificity of the inhibition of cdk2 activity was determined. Cdk2 binds cyclin D1 and cyclin E during the G1 phase and G1 to S phase transition, respectively (reviewed in Pines and Hunter, 1995). Cell lysates from DU145 cells treated with IFNα for 16 or 24 hours were immunoprecipitated using antibodies specific for cyclin D1 or cyclin E and cdk2 activity was subsequently assessed (Table IX). IFNα treated cells showed up to a 38% reduction of cyclin E-cdk2 activity over the control, but did not show consistent inhibition of cyclin D1-cdk2 activity. Of the 74% IFNα induced reduction in overall cdk2 activity (Table VIII), 38% is apparently due to the reduction of cyclin E dependent cdk2 activity (Table IX). The remaining inhibition of cdk2 activity by IFNα is probably due, at least in part, to inhibition of activity in the cyclin A-cdk2 complex (Tiefenbrun et al., 1996). I next immunoprecipitated cyclin E and immunoblotted using a cdk2 antibody in order to determine relative amounts of cdk2 complexed to cyclin E. Figure 7 shows that IFNα treatment of cells did not affect the levels of cdk2 complexed with cyclin E. Consequently, IFNα inhibition of the activity of the cyclin E-cdk2 complex did not affect the formation of the cyclin E-cdk2 complex in DU145 cells.

Induction of CKI $p21^{WAF1}$

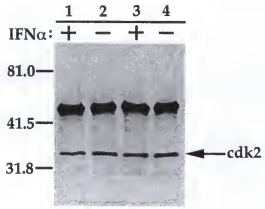
The IFNα induced decrease in cyclin E-cdk2 activity suggested that a kinase inhibitor may be involved. The CKI p21 is known to bind cyclin E-

Table IX: Effect of IFNα treatment on cyclin specific cdk2 activity[a]

Cyclin	Time (hours)	IFNα (units/ml)	CPM	reduction (%)
Expt. 1				
E	16	0	29507	--
		2500	22861	22.5
	24	0	28607	--
		2500	17742	37.9
D1	16	0	5321	--
		2500	3881	27.1
	24	0	4642	--
		2500	5291	--
Expt. 2				
E	16	0	15954	--
		2500	11761	26.3
	24	0	14296	--
		2500	9907	30.1
D1	16	0	4786	--
		2500	4936	--
	24	0	5666	--
		2500	7818	--

[a]DU145 cells were treated with 0 or 2500 units/ml IFNα for 16 or 24 hours. The cyclin-cdk2 complex was immunoprecipitated with antibodies specific for either cyclin E or cyclin D1. Cdk2 activity was then assessed by histone H1-dependent kinase activity. Cdk2 activity is represented as cpm with corresponding percent reduction. Similar data with the same patterns were observed in two repeats of this experiment.

Figure 7. IFNα does not affect cyclin E-cdk2 complex formation in DU145 cells. The presence of cdk2 complexed with cyclin E was assessed by immunoprecipitation of DU145 cell lysates (515 μg protein/ 500 μl for each sample) with cyclin E antibodies and immunoblotting with antibodies specific for cdk2, as described in 'Materials and Methods'. Lanes 1 and 2 represent lysates from cells treated for 16 hours with 2500 units/ml IFNα or medium alone, respectively. Lanes 3 and 4 are from cells treated for 24 hours with or without IFN, respectively.

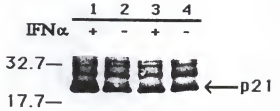

1 2 3 4
IFNα: + − + −
81.0
41.5
31.8
cdk2

cdk2 and block its activity (reviewed in Pines and Hunter, 1995). Serum starvation of cells increases p21 levels and restimulation by serum a reduction in p21 levels (Pines and Hunter, 1995). DU145 cells synchronized by serum starvation into G0/G1 were therefore used to determine whether p21 is involved in inhibiting the G1 to S phase transition in IFN treated cells. Cell lysates from DU145 cells treated with IFNα for 16 and 24 hours were immunoprecipitated using p21 antibodies, and the presence of both p21 and cdk2 in these samples was subsequently assessed by immunoblotting. As shown in Figure 8A, lysates from cells treated with IFNα appeared to have a greater expression of p21 than untreated cells. In the same immunoprecipitates, at 16 and 24 hours, cdk2 protein levels were also higher in IFNα treated cells than in untreated cells (Figure 8B), supporting the conclusion that in IFNα treated cells increased levels of p21 were associated with cdk2. This suggests that the expression of p21 in IFNα treated cells played an important role in inhibiting the cdk2 activity in these cells.

To establish that IFNα treatment induces the expression of p21, cell lysates from asynchronous DU145 cells that were treated with IFNα or medium alone were assessed for p21 as described above. IFNα treatment progressively induced the expression p21 (Figure 9A) over that seen in untreated cells. Densitometric analysis (Figure 9B) of the p21 bands in Figure 9A showed that IFN treated cells had approximately twice the levels of p21 compared to untreated cells. Thus, IFNα inhibits the G1 to S phase transition of the cell cycle by inducing p21 expression in a prostate cancer cells. Similar

Figure 8. A. IFNα treatment increases and/or maintains p21 levels in synchronized DU145 cells. Cell lysates (512 µg protein/ 500 µl for each sample) were immunoprecipitated and immunoblotted using p21 antibodies, as described in 'Materials and Methods'. Lanes 1 and 2 represent lysates from synchronized cells treated with 2500 U/ml IFNα or media alone, respectively, for 16 hours. Lanes 3 and 4 are from cells treated for 24 hours with or without IFN, respectively. The percent decrease in p21 levels for lanes 1, 2, 3, and 4 was 40.1%, 62.7%, 68.2%, and 70%, respectively, as determiend using densitometric scanning of radiographic film. The percent decrease represents the ratios of band intensities from DU145 cell lysates at initiation of cultures (0 hours, data not shown) and lanes 1 through 4. B. Cdk2 levels correspond to p21 expression in IFNα treated cells. The immunoblot from Figure 5A was reanalyzed using antibodies specific for cdk2. The lane assignments are as stated for 5A. The percent decrease in cdk2 protein levels was 64.3%, 81.5%, 83.2%, and 91% for lanes 1, 2, 3, and 4 respectively, as determined by densitometric scanning as described for A.

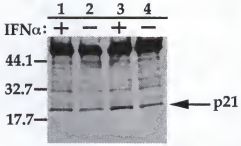
A.



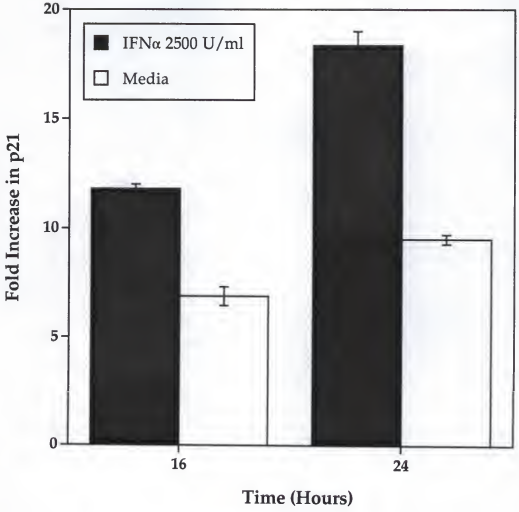
B.

Figure 9. A. IFNα induces p21 expression in DU145 cells. Cell lysates from DU145 cells (486 µg protein/ 500 µl for each sample) were immunopreciptated and immunoblotted with antibodies specific for p21. Lanes 1 and 2 represent lysates from cells treated for 16 hours with 2500 U/ml IFNα or media alone, respectively. Lanes 3 and 4 represent lysates from cells treated for 24 hours with or without IFN, respectively. B. The fold increase in p21 levels for 16 and 24 hours in IFN treated and untreated cells was determined by densitometric scanning of radiographic film. The fold increase represents the ratios of band intensities from DU145 cell lysates at initiation of cultures (0 hours, data not shown) and Lanes 1 through 4. Standard deviations represent three separate densitometric readings from figure 9A.

A.


1 2 3 4
IFNα: + − + −
44.1
32.7
17.7
p21

B.


IFNα 2500 U/ml
Media
Fold Increase in p21
20
15
10
5
0
16
24
Time (Hours)

experiments looked at the possible roles of two other CKIs, p27 and p16. Induction of p27 or p16 expression was not seen with IFN treatment of DU145 cells (data not shown) suggesting a unique role for p21 in these effects. IFNα inhibits the G1 to S phase transition of the cell cycle by inducing p21 expression in a prostate cancer cell line.

IFNγ Inhibition of the DU145 Cell Cycle

Inhibition of the Cell Cycle

Studies using IFNγ were begun by determining the antiproliferative effects of IFNγ on DU145 cells. IFNγ was able to inhibit colony formation of DU145 cells at low cell density in a dose dependent manner as shown in Table X. Treatment of DU145 cells with IFNγ showed significant inhibition of colony formation at concentrations as low as 312 U/ml with up to 70% inhibition at 10,000 U/ml. The effects of IFNγ on the DU145 cell cycle were then analyzed using flow cytometry (Table XI). DU145 cells synchronized by serum starvation into G0/G1 were stimulated to enter the cell cycle by serum addition in the presence or absence of 2500 U/ml IFNγ. Untreated cells rapidly advanced through the G0/G1 and S phases of the cell cycle, and, by 24 hours, these cells began returning to the G1. IFNγ treated cells, however, progressed more slowly. At 16 hours, 46% of the IFN treated cells remained in G1 compared to only 33% of the untreated cells. By 24 hours, in contrast to the untreated cells, the largest percentage of IFN treated cells were in the S phase. A similar pattern was seen with 1000 U/ml IFNγ (data not shown).

Table X: Inhibition of DU145 colony formation by IFNγ [a]

IFNγ (units/ml)	colonies/well (mean±S.D.)	Inhibition (%)
0	110.5 ± 8.7	---
312	74.3 ± 3.8	32.6
625	62.7 ± 3.1	43.8
1250	57.3 ± 3.2	48.1
2500	51.3 ± 4.0	53.6
5000	41.7 ± 1.5	62.3
10,000	33.7 ± 3.1	70.6

[a]DU145 cells were plated at 800 cells/well with various doses (units/ml) of IFNγ for 5 days and subsequently stained with crystal violet. Samples were assessed in triplicate and results are expressed as the mean number of colonies ± S.D. Statistical significance was shown by Student's t-test between the number of colonies in the presence or absence of 312 U/ml ($p<0.05$), 625 U/ml ($p<0.02$), 1250 U/ml ($p<0.01$), 2500 U/ml ($p<0.01$), 5000 U/ml ($p<0.005$), and 10,000 U/ml ($p<0.005$) of IFNγ.

Table XI: Effects of IFNγ on the DU145 cell cycle[a]

Time (hours)	IFNγ (units/ml)	cell cycle phase (%)		
		G0/G1	S	G2/M
0	0	74.1	15.3	10.6
16	0	33.3	60.5	6.2
	2500	45.6	44.3	10.0
20	0	26.9	53.6	19.5
	2500	32.0	55.9	12.2
24	0	36.8	33.6	29.6
	2500	34.1	40.7	25.2

[a]DU145 cells were treated with 0 or 2500 units/ml of IFNγ for 0, 16, 20, or 24 hours. Progression through the cell cycle was examined using propidium iodide staining. Data from 30,000 events are presented as percentage of cells in each stage of the cell cycle. Similar results were seen with two replicates of this experiment.

IFNγ inhibits the proliferation of DU145 cells by slowing their progression through the cell cycle.

Induction of $p21^{WAF1}$

As previously mentioned, p21 is known to act at the G1 and S phases of the mammalian cell cycle by directly binding to cdk-cyclin complexes that are active at these stages and blocking their activity (Gartel et al., 1997). To determine whether IFNγ treatment induces p21 in DU145 cells, cell lysates from asynchronous cells that were treated with IFNγ or media alone were assessed for p21 by immunoblotting (Figure 10). At the initiation of treatment, rapidly dividing cells exhibited low levels of p21. After 20 and 40 hours of IFNγ treatment, p21 levels appeared to be greatly increased over that of the untreated cells. As seen with IFNα, this induction of p21 by IFNγ was unique since other cdk inhibitors such as p27 and p16 did not show similar increases in response to IFN treatment (data not shown).

$p21^{WAF1}$ Induction Causes an Increase in p21 Bound Cdk2 and PCNA

I further assessed the consequences of the induction of p21 by IFNγ using immunoprecipitation. Using antibodies specific for p21, cell lysates from DU145 cells treated with medium alone or IFNγ were immunoprecipitated. These precipitates were then immunoblotted using antibodies specific for p21, cdk2, and PCNA. Consistent with previous studies on the role of p21 in the cell cycle, cdk2 and PCNA levels correlated with the

Figure 10. IFNγ induces p21 expression in DU145 cells. DU145 cell lysates (141 μg protein per lane) were immunoblotted with antibodies specific for p21. Lane 1 represents cell lysate from dividing cells at the initiation of treatment (time 0), lanes 2 and 3 are lysates from cells treated for 20 hours with 2500 units/ml IFNγ or media alone, respectively, and lanes 4 and 5 are lysates from cells treated 40 hours with or without IFNγ, respectively. Densitometric analysis of radiographic film showed a 2.1-fold and 2.6-fold difference in p21 levels between lanes 2 and 3 and lanes 4 and 5, respectively.

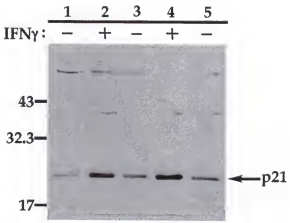
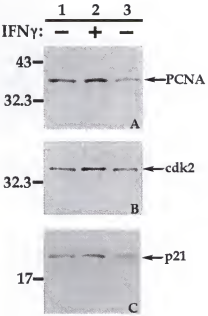

1 2 3 4 5
IFNγ: − + − + −
43
32.3
17
p21

Figure 11. Cdk2 and PCNA levels correspond to p21 induction by IFNγ. DU145 cell lysates (463 μg protein/ 500 μl for each sample) were immunoprecipitated using antibodies specific for p21 and subsequently immunoblotted using antibodies for (a) PCNA, (b) cdk2, and (c) p21. For (a), (b), and (c), lane 1 represents lysates from untreated cells at initiation of IFN treatment while lanes 2 and 3 are from cells treated with 2500 units/ml IFNγ or media alone for 30 hours, respectively. Densitometric analysis of radiographic film showed a 6.3-fold difference in PCNA levels (a), a 2.22-fold difference in cdk2 levels (b), and 2.7-fold difference in p21 levels (c) between lanes 2 and 3.


1 2 3
IFNγ: − + −
43
32.3
PCNA
A
32.3
cdk2
B
17
p21
C

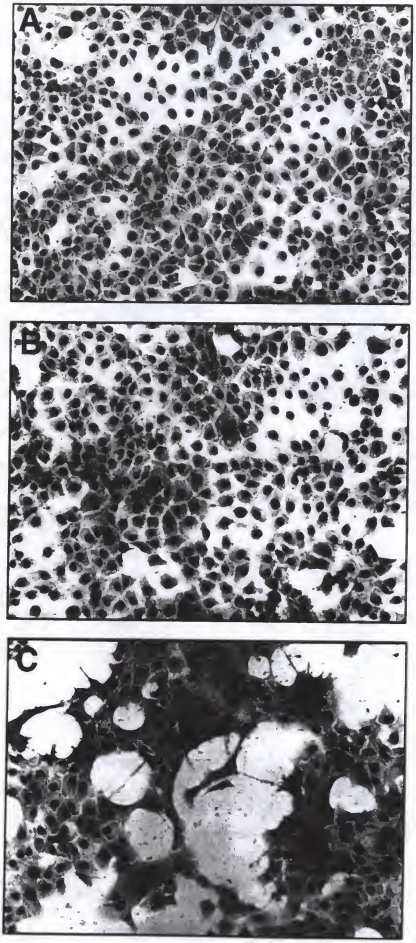
level of p21 expression (Figure 11). The increase in p21 resulting from IFNγ treatment corresponded to an increase in p21 bound cdk2 and PCNA. Cdk2 is a cyclin dependent kinase which is active in the G1 and S phases of the cell cycle, while PCNA is an essential component of the DNA replication machinery (Gartel et al., 1997; Kelman, 1997). By binding these proteins, p21 acts to inhibit cell replication, and the induction of p21 by IFNγ suggests that these aspects of cellular replication are inhibited. Based on the previous studies with IFNα, both type I and type II IFNs appear to block the DU145 cell cycle at the G1 and S phases via a similar mechanism of p21 induction.

IFNγ Induction of a Change in Cell Phenotype

Changes in Cell Morphology

In the course of studies on the effects of IFNs on the cell cycle, it was observed that IFNγ treatment induced a change in the appearance of DU145 cells. I therefore looked at the impact of IFNα and IFNγ on DU145 cellular morphology. DU145 cells were treated with medium alone, IFNα, or IFNγ for 5 days, subsequently fixed, and stained with eosin-methylene blue. Analysis by light microscopy showed that while untreated and IFNα treated cells retained normal tissue culture appearance, IFNγ treated cells showed a distinct morphological change (Figure 12). Untreated and IFNα treated cells exhibited a rounded morphology typical of tumor cells growing in culture. IFNγ treated cells were less rounded with protuberances resulting in more of a spindle shape. Changes in cellular morphology are often associated with a

Figure 12. IFNγ induces morphological changes in DU145 cells. DU145 cells were treated with (a) medium alone, (b) 5000 units/ml IFNα, or (c) 5000 units/ml IFNγ for 96 hours, subsequently stained with eosin-methylene blue, and analyzed by light microscopy. Total magnification is 100X.

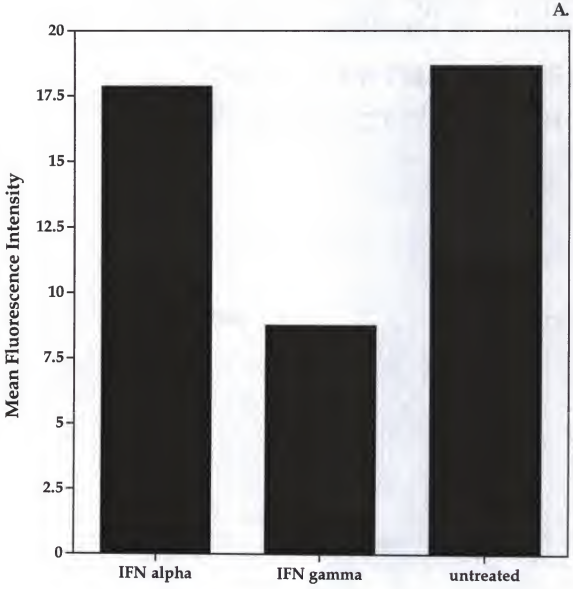

A
B
C

change in cellular phenotype and differentiation. The DU145 cell line displays many features common to undifferentiated metastatic tumor cells, including loss of several functional tumor suppressor gene products as well as being tumorigenic in nude mice (Bookstein et al., 1990; Dong et al., 1995; Isaacs et al., 1991). The effects of IFNγ on the morphology of DU145 cells suggests that IFNγ induces a phenotypic change and possibly differentiation of these cells.

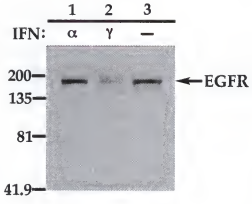
Downregulation of the EGF Receptor

Expression of the EGFR and its ligands has been associated with human cancer cells of various origins and, in several instances, has been correlated with the stage of differentiation of tumor cells (Cohen et al., 1994; Ioachim et al., 1996; Nakopoulou et al., 1995; Stumm et al., 1996). Further, in some malignancies, increased EGFR expression has been found to be an indicator of poor patient prognosis (Almadori et al., 1995; Kristensen et al., 1996). The DU145 cell line has previously been shown to express EGFR, as well as its ligands EGF and TGFα, and, as a result, possesses an autocrine feedback loop (Connolly and Rose, 1991). I looked at the effects of type I and type II IFNs on the expression of the EGFR. DU145 cells were treated with medium alone, IFNα, or IFNγ for 5 days and the expression of the EGFR was analyzed by flow cytometry using antibodies specific for the EGFR (Figure 13A). IFNγ caused greater than a 50% reduction in EGFR compared to untreated cells. No effect on EGFR expression was seen with IFNα treatment. These effects were also

Figure 13. IFNγ downregulates the expression of the EGF receptor. DU145 cells were treated with medium alone, 5000 units/ml IFNα, or 5000 units/ml IFNγ for 5 days. (a) Cells were stained using antibodies specific for the EGFR and analyzed by flow cytometry. Similar data with the same pattern were observed in 3 repeats of this experiment. Data are expressed as mean fluorescence intensity. (b) DU145 cell lysates (138 μg protein per lane) were immunoblotted using antibodies specific for EGFR. Lane 1 represents lysate from cells treated with IFNα, lane 2 is from cells treated with IFNγ, and lane 3 is from untreated cells. Densitometric analysis of radiographic film showed a 80% decrease in EGFR levels between IFNγ and untreated cells (lanes 2 and 3).


A.
Mean Fluorescence Intensity
20
17.5
15
12.5
10
7.5
5
2.5
0
IFN alpha
IFN gamma
untreated

B.



analyzed by western blot (Figure 13B). Again, while no change was seen with IFNα, IFNγ clearly downregulated the expression of EGFR. I then looked at the expression of the EGFR ligand EGF (Figure 14). Both IFNα and IFNγ reduced EGF production with IFNα showing the more significant downregulation. These results are interesting in that both IFNα and IFNγ reduced growth factor production and cellular replication, but only IFNγ affected the receptor expression.

To determine the significance of the decrease in receptor expression, the impact of EGF on cells pretreated with IFNα and IFNγ was examined. EGF is known to stimulate quiescent cells to enter the cell cycle and increase cyclin D1 expression (reviewed in Lavoie et al., 1996). Cyclin D1 is a proto-oncogene that has been found to be overexpressed in a number of human neoplasms (Lees and Harlow, 1995). Lysates from IFN treated or untreated cells stimulated with EGF were assessed for cyclin D1 expression. Figure 15 shows that IFNγ treated cells exhibited little expression of cyclin D1 even with the addition of EGF to the cell culture medium. In contrast, IFNα treated cells expressed higher levels of cyclin D1 and showed a modest increase in the presence of EGF. A similar pattern was seen with untreated cells. Further, IFNα treated cells in the presence of EGF increased in number by thirty percent over those without EGF while no difference in cell number was seen with IFNγ and EGF (data not shown). These findings indicate that the downregulation of EGFR expression on DU145 cells by IFNγ results in a cell type that is less sensitive to the growth enhancing effects of EGF. Previous

Figure 14. IFNα and IFNγ reduce EGF production by DU145 cells. DU145 cells were treated with medium alone, 5000 units/ml IFNα, or 5000 units/ml IFNγ for 5 days. Cells were then harvested and reseeded into 6 well plates at 1×10^5 cells/well. Supernatants were collected at 24 hours and analyzed by ELISA for EGF. Data are expressed as mean concentration ± S.D. Statistical significance was shown by Student's t-test between m.f.i. for cells treated with media alone and IFNα ($p<0.02$).

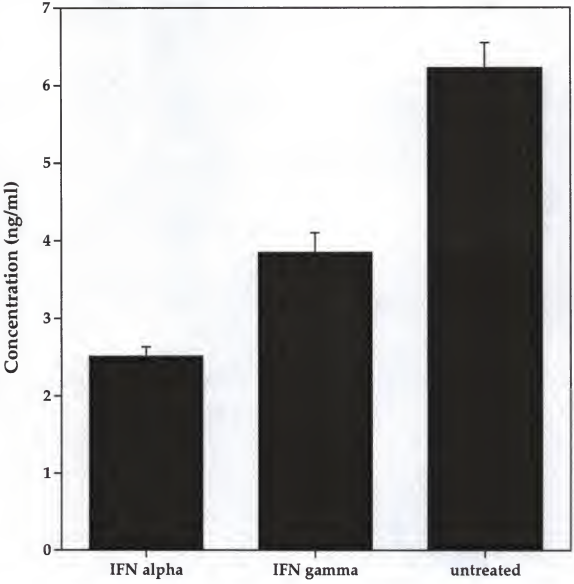
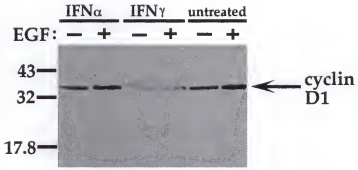

Concentration (ng/ml)
7
6
5
4
3
2
1
0
IFN alpha
IFN gamma
untreated

Figure 15. EGF does not induce cyclin D1 in IFNγ treated cells. DU145 cells were treated for 5 days with either 5000 U/ml IFNα, 5000 U/ml IFNγ or medium alone followed by the addition of 0.5 ng/ml EGF to fresh culture media and incubated for 6 hours. Cell lysates (155 μg protein per lane) were then immunoblotted for the presence of cyclin D1. Densitometric analysis of radiographic film showed a 31% increase between lanes 1 and 2, a 6% increase between lanes 3 and 4, and a 23% increase between lanes 5 and 6 of cyclin D1 levels. In addition, there is a 61% increase in cyclin D1 between lanes 3 (IFNγ) and 5 (untreated).


IFNα
IFNγ
untreated
EGF: – + – + – +
43
32
17.8
cyclin
D1

studies have shown that in vitro invasiveness of DU145 cells and in vivo progression of DU145 tumors in nude mice are modulated by EGFR mediated signals (Prewett et al., 1996; Xie et al., 1995). The downregulation of EGFR by IFNγ in DU145 cells suggests that IFNγ induces a less malignant phenotype and decreases the invasive potential of this cell line.

Modulation of Adhesion Molecules

In addition to soluble factors, cell-cell and cell-extracellular matrix (ECM) adhesion properties underlie many of the phenotypic changes associated with tumor progression and metastatic potential (Hannigan and Dedhar, 1997). The effects of IFNγ on the expression of the adhesion molecules ICAM-1 and integrin α3 were analyzed (Table XII). The ability to evade the host immune response is characteristic of tumor cells. ICAM-1 plays a role in leukocyte adhesion by binding the LFA-1 surface receptor found on cytotoxic T lymphocytes (CTL), B cells, and natural killer (NK) cells (Dana and Arnaout, 1994; Makgoba et al., 1988). As a result, cells with high ICAM-1 expression could be the target of a host immune response in vivo. ICAM-1 has previously been found to be an immediate response gene induced by IFNγ (Caldenhoven et al., 1994). IFNγ was found to induce ICAM-1 expression in DU145 cells by 239% over that of untreated cells. An increase in integrin α3 expression was also seen as a result of IFNγ treatment (Table XII). Integrins mediate interactions between cells and ECM, and previous work with another prostate carcinoma cell line, PC-3, has shown that highly

Table XII: Effects of IFNα and IFNγ on the expression of ICAM-1 and integrin α3[a]

	ICAM-1		Integrin α3	
	m.f.i.±S.D.	increase (%)	m.f.i.±S.D.	increase (%)
untreated	153.2 ± 4.2	---	368.2 ± 9.9	---
IFNα	127.2 ± 2.8	---	387.7 ± 1.0	5.3%
IFNγ	519.7 ± 26	239%	457.2 ± 12.7	24.2%

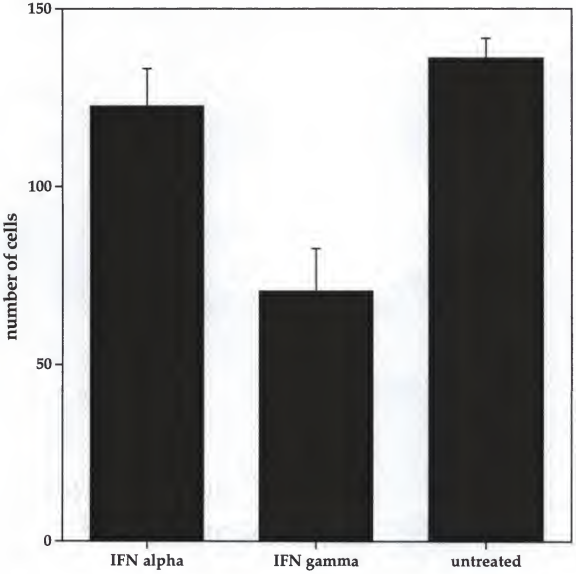
[a]DU145 cells were treated with either 5000 units/ml IFNα, 5000 units/ml IFNγ, or media alone for 5 days. Cells were stained with either antibodies specific for ICAM-1 or integrin α3 and analyzed by flow cytometry. Data are expressed as mean fluorescence intensity (m.f.i.)± S.D for three replicates. Statistical significance was shown by Student's *t*-test between m.f.i. for cells treated with IFNγ or media alone for ICAM-1 ($p<0.03$) and integrin α3 ($p<0.01$).

invasive PC-3 variants have low levels of integrin α3 expression (Dedhar et al., 1993). Thus, upregulation of the ICAM-1 and integrin α3 adhesion molecules suggests that IFNγ induces a less tumorigenic and less metastatic phenotype in DU145 cells.

Reduction in Invasive Potential

I next tested whether the modulation of surface receptors on DU145 cells actually correlated with a change in invasive potential. Invasion by tumor cells is a crucial step in the multistage process of tumor spread and the formation of metastasis (Liotta, 1987). Several in vitro systems have been established to study the invasiveness of tumor cells. One commonly used system utilizes insert chambers with separating filters coated with a layer basement membrane matrix that contains components similar to the ECM. These invasion chambers are suitable for studying cell invasion of malignant cells (Albini et al., 1987). DU145 cells were treated for 5 days with or without IFN. These cells were subsequently reseeded into invasion chambers which contain membranes coated with a basement membrane matrix. The total number of cells which migrated across the membrane were then determined for each treatment group (Figure 15). IFNγ treated cells showed a 50% reduction in the number of invasive cells over that of the control. Furthermore, only a slight reduction in invasion of IFNα treated cells was seen. Thus, the reduction in the number of invasive cells for the DU145 cell line was specific for IFNγ compared to IFNα even though both IFNs inhibited

Figure 16. IFNγ decreases the invasive potential of DU145 cells. DU145 cells were treated with medium alone, 5000 units/ml IFNα, or 5000 units/ml IFNγ for 5 days. Cells were then reseeded into invasion chamber inserts containing matrigel basement membrane matrix. Cells that migrated across the matrix were stained with eosin-methylene blue and counted. Data are expressed as the mean number of cells ± S.D for three replicates.


150
100
50
0
number of cells
IFN alpha
IFN gamma
untreated

cell growth. These results imply that IFNγ can both reduce cell growth and metastasis of DU145 prostate cancer cells.

CHAPTER 4
DISCUSSION

Both type I and type II IFNs possess potent antitumor properties for a variety of cell types by inducing a number of cellular responses. Previous studies have looked at the antiproliferative effects of IFNs on cancer cells both in vivo and in vitro, and have shown that IFNs directly act on tumor cells to prolong their progression through the cell cycle (Fleischman and Fleischman, 1992). Indirect antitumor functions of IFNs include the activation of different aspects of the immune system, which leads to the targeting of cancer cells by cytolytic immune cells (Baron et al., 1991). The combination of these antitumor properties has led to the use of IFNs in a number of human malignancies. However, while IFNs are therapeutic with certain cancers, they are not with some others. Many of the cases where IFNs have not been found to be clinically useful may be a result of inadequate knowledge of the mechanisms by which IFNs function. The mechanisms behind the antiproliferative effects of IFNs are poorly understood. Previous work has shown with some cell types IFNs inhibit the G1 and S phases of the cell cycle (Creasey et al., 1980; Pontzer et al., 1991; Roos et al., 1984; Tamm et al., 1987) and modulate the expression of c-myc and pRB (Einat et al., 1985a; Einat et al., 1985b; Jonak and Knight, 1984; Melamed et al., 1993). The vast majority of these studies have been carried out on the Daudi cell line, which is a B cell

lymphoma. Daudi cells are extremely sensitive to the antiproliferative effects of IFNα and undergo G1 arrest upon treatment with IFN (reviewed in Subramaniam et al., 1997). Few studies have used cells of other origins to study the effects of IFNs on the cell cycle.

The DU145 cell line, as previously mentioned, is an interesting model to study the mechanism of the regulation of the cell cycle by IFNs. These cells were isolated from a metastatic lesion and are a poorly differentiated adenocarcinoma (Stone et al., 1978). Most malignancies, including prostate cancer, with high metastatic potential and poor differentiation of cells are associated with poor patient prognosis (Garnick, 1994). Tumors of the prostate that are well-differentiated are more likely to be confined to the prostate gland and can be treated by removal of the prostate (Garnick, 1994). A poorly differentiated prostatic adenocarcinoma is more likely to extend beyond the prostate and metastasize. Currently, besides radiation, hormonal therapy is the only common treatment of prostate cancer once metastasis has occurred (Garnick, 1994).. The drawback to this therapy is that, at some point, most metastatic tumors become resistant to hormonal therapy and, as a result, progress rapidly. By studying the DU145 cell line, we can gain insight into the mechanism of IFNs on a metastatic adenocarcinoma.

DU145 cells are sensitive to the antiproliferative effects of IFNα and IFNγ, and, consistent with previous studies on other cell lines, appear to be affected at the G1 to S phase transition of the cell cycle. This inhibition of cell replication is at least partly due to an increase in p21 expression and the

subsequent decrease in activity of the cyclin E-cdk2 complex. The interaction of p21 with cyclin E-cdk2 blocks the phosphorylating activity of this complex which is necessary for the G1 to S phase transition of the cell cycle and DNA replication (Gartel et al., 1997; Pines and Hunter, 1995; Sherr, 1993). p21 also inhibits DNA replication by binding PCNA (Kelman, 1997). By inducing p21 expression, IFNs are able to control different aspects of the cell cycle. These results describe a possible mechanism for the antiproliferative affects of IFNs on a prostate cancer cell line.

Many of the proteins involved in controlling the cell cycle have been designated as products of tumor suppressor genes or oncogenes due to the association of mutations of these genes with different types of cancers. DU145 cells have mutations in the tumor suppressor gene products p53, pRB, PTEN, and KAI1 (Bookstein et al., 1990; Dong et al., 1995; Isaacs et al., 1991; Li et al., 1997). Various oncogenes and tumor suppressor genes have been identified in prostatic tumors including mutations in p53 and pRB (Netto and Humphrey, 1994). This provides another characteristic of DU145 cells that makes them interesting to study. Approximately fifty percent of all cancers have mutations in the p53 genes, and pRB is frequently deregulated in a variety of tumors (reviewed in Gangopadhyay et al., 1997; La Thangue, 1997). The mutant p53 in DU145 cells means that, although p53 is known to induce p21, the induction of p21 by IFNs in this case is independent of p53 status. The pRB gene product is directly phosphorylated by cyclin E-cdk2 complexes that are inhibited by p21 (Hatakeyama and Weinberg, 1995). Again, in the case

of DU145 cells, this aspect of p21 regulation of the cell cycle does not contribute significantly to the inhibition of cell replication, because of the pRB mutation. Other features of p21 regulation, including the binding of PCNA and other potential cdk2 targets, are therefore most likely involved.

Although both IFNα and IFNγ can induce p21, IFNγ induced biochemical changes associated with changes in cellular phenotype indicate that it possesses other antitumor effects against DU145 cells not related to p21. One significant effect that appears to be independent of p21 status is the downregulation of the EGFR. The EGFR is the most studied of the tyrosine kinase receptors and has long been associated with human tumorigenesis (Helden and Ronnstrand, 1997). It is frequently found to be overexpressed or mutated in several different human tumor types. Like other cells of epidermal origin, prostate cells are stimulated by EGF to replicate. The DU145 cell line in particular has been shown to have an autocrine stimulatory loop with the EGFR and its ligands (Connolly and Rose, 1991). Therefore, IFNγ regulation of the EGFR lends insight into the mechanism behind IFNγ control of cell replication and phenotype. Other surface receptors modulated by IFNγ, and not IFNα, are ICAM-1 and integrin α3. Integrin α3 has been correlated with the metastatic potential of another prostatic adenocarcinoma cell line PC-3. The direct involvement of the EGFR and integrin α3 in the invasiveness of these cells remains to be elucidated. However, they could act as important markers of a metastatic phenotype. The strong upregulation of the ICAM-1 adhesion molecule by IFNγ is another aspect of its antitumor

effects. An increase in ICAM-1 could allow these cells to be more easily targeted by the immune system. ICAM-1 is the receptor for the integrin LFA-1 which is found on the surface of CTLs, NK cells, and B cells (Dana and Arnaout, 1994; Makgoba et al., 1988). The significance of this increase in cell-cell interactions may be seen with in vivo metastasis. IFNγ not only reduces the invasiveness of the DU145 cells, but the few cells that do escape the tumor site could be more readily attacked by the host immune system.

As stated above, since both IFNα and IFNγ upregulate p21, the phenotypic changes that result from IFNγ must be independent of p21. It is likely that IFNs influence multiple biochemical pathways that may or may not overlap. Although neither IFNα or IFNγ induced the CKIs p27 or p16, it is possible that IFNs can affect other regulators and inhibitors that influence tumor cell growth. Previous studies with Daudi cells have shown that IFNs suppress the phosphorylation of pRB (Melamed et al., 1993). Although this modulation of pRB is insignificant in DU145 cells, the cell cycle is still inhibited. The ability of IFNs to affect multiple cellular pathways in order to regulate cell growth allows for the potential of IFNs to affect a wide range of cell types with various genetic mutations. For example, regulation of the EGF production is significant for the regulation of adenocarcinomas as well as cancers of other origins due to the number of genes and biochemical pathways stimulated by the EGFR signal transduction. Based on the effects of IFNs on DU145 cells, cancers with mutations in p21 and/or the EGFR, such as the erb-B family of oncogenes, may not be the best candidates for IFN therapy.

Determining the mechanism behind IFNs antitumor capabilities is also useful for finding potential combination therapies. By understanding the pathways influenced by IFNs, cytotoxic agents that can produce synergistic effects when combined with IFNs may be found.

Even though both IFNα and IFNγ inhibit DU145 cell growth, the phenotypic changes induced by IFNγ suggest that it is superior to IFNα as an antitumor agent for DU145 prostate cancer cells. This is in contrast to studies carried out using the Daudi cell line (Subramaniam et al., in press). While IFNα induces a strong G1 arrest, IFNγ has no antiproliferative effects on Daudi cells. There are several potential explanations for the differential effects of IFNs on various cell lines. Possibilities include IFN receptor expression, signal transduction pathways, and target genes. For this reason, studies into the mechanism behind the cellular actions of IFNs are relevant.

The characterization of the antitumor effects of IFNs on DU145 cells gives insight into the poorly defined mechanism of IFN antiproliferative and antitumor functions. The DU145 cell line is a metastatic adenocarcinoma with mutations in key tumor suppressor genes that are commonly defective in a variety of human cancers. Even with these mutations and metastatic potential, both IFNα and IFNγ are able to exert potent antiproliferative activities, with IFNγ also inducing a phenotypic change that results in an antitumor effect. Future studies will include further defining the mechanism of IFNγ effects on DU145 cells. These will include determining how IFNγ controls the expression of the EGFR. In addition, the possible involvement of

collagenase activity in the IFNγ reduction of the DU145 invasive potential, via the downregulation of the EGFR, may be determined. Finally, work with nude mice will determine the effects on IFNγ on the metastatic potential of DU145 cells using an in vivo system. These studies will expand on the work contained in this dissertation and provide further details of the mode of action of IFNs on an adenocarcinoma cell line.

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BIOGRAPHICAL SKETCH

Amy Claudine Hobeika, a first-generation American, was born in Cincinnati, Ohio, on December 10, 1970, to Claude and Terry Hobeika. Her father Claude Hobeika was born and raised in Alexandria, Egypt and came to the United States in 1959 to study medicine. While studying in Lexington, KY, he met a nursing student named Terry Taphorn, and they married in 1961. In 1963, they moved to Gainesville, Florida, where Dr. Hobeika became a resident at the University of Florida teaching hospital. Amy is the youngest of four children. Her sister Janine is the eldest and her two brothers John and Claude are in the middle. Amy attended Ann Weigel Elementary School and Seven Hills Middle and Upper Schools while growing up in Cincinnati, although spending her vacation time to a large extent in Sarasota, Florida. In 1988, she graduated from high school, and much to her fathers dismay, attended college at the University of the South in Sewanee, Tennessee, instead of at the University of Florida. After a rocky freshman year, she decided to study a field that actually held her interest and majored in biology. In 1992, Amy recieved her Bachelor of Science and, to her fathers great joy, moved to Gainesville, Florida, to further her education. In 1993, she was accepted into the Department of Microbiology and Cell Science where she was kindly taken into the laboratory of Dr. Howard Johnson. After graduation, Amy will remain in the Johnson laboratory as a postdoctoral fellow until her

"significant other", Adrian, finishes medical school. Then, hopefully, it is on to a successful future.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Howard M. Johnson, Chair
Graduate Research Professor of
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Microbiology and
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Associate Professor of
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1997

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